AWARD NUMBER: W81XWH-13-1-0386

TITLE: Imaging Prostate Cancer (Pca) Phenotype and Evolution

PRINCIPAL INVESTIGATOR: Jason A. Koutcher

CONTRACTING ORGANIZATION: SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH
                                         New York, NY 10065

REPORT DATE: October 2014

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                         Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2014	Annual	30 Sep 2013 – 29 Sep 2014

4. TITLE AND SUBTITLE

Imaging Prostate Cancer (Pca) Phenotype and Evolution

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-1-0386

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Jason Koutcher

E-Mail:koutchej@mskcc.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH

New York, NY 10065

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The goal of the project is to investigate the potential of inhibiting iron metabolism to inhibit prostate cancer growth. Specifically, we will study Deferiprone, an iron chelator, and focus on its effect on aconitase in prostate tumors. It has been shown that changes in citrate metabolism at the level of mitochondrial aconitase, is an early change in carcinogenesis in the prostate. This change in metabolism is detectable by magnetic resonance. The project includes both in vitro and in vivo studies to determine its potential utility for clinical translation. Our findings to date include 1) showing that Deferiprone inhibits tumor growth in 2 prostate cell lines, 2) demonstrated an effect on metabolism in perfused cells (one cell line), and 3) confirmed changes in metabolism by measuring changes in oxygen consumption rate. After 24 hours of incubation, deferiprone treated TRAMP C2 cells also showed lower oxygen consumption rates (OCR) and down regulation of mitochondrial aconitase expression, leading to lower enzyme activity as detected in both TRAMP C2 and Myc-Cap cell lines. The effects on both metabolism and proliferation are encouraging and we hope in Year 2 to begin in vivo studies and complete in vitro studies

15. SUBJECT TERMS					
Deferiprone, aconitase, metabolism, tricarboxylic acid cycle, magnetic resonance					

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE	Unclassified	11	19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified			

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

3

Table of Contents

1. **Introduction:** Normal prostate peripheral tissue has low mitochondrial aconitase (m-Acon) activity. This has been associated with zinc-induced inhibition of m-Acon in the peripheral epithelial cells. Activation of m-Acon is an early biochemical change during prostate cancer development and has been associated with a down-regulation of zinc transporters. This leads to a shift from citrate-producing to a citrate-oxidizing malignant phenotype, which has been extensively observed in different human prostate cancer cell lines. Thus, in the clinical setting, high levels of citrate are typically observed in normal prostate epithelia while essentially non-detectable in prostate cancer tissue, particularly in high grade tumors.

Aconitase is an iron-sulphur enzyme that catalyzes the two-step isomerization of citrate to isocitrate, and has two isoforms: m-Acon (mitochondrial aconitase) and c-Acon (cytoplasmic; additionally functions as iron regulatory protein 1, when the iron levels in the cell become low). The unique $[Fe_4S_4]^{2+}$ cluster of aconitase reacts directly with the substrate and contains a labile iron atom, which must be replaced occasionally. Thus, when the iron levels in the cell become low, the cluster cannot be regenerated and the enzyme becomes inactive: $[Fe_3S_4]^+$. In fact, iron is present in many enzymes and is essential for cancer metabolism, cell growth, and proliferation and thus has been suggested as a potential chemotherapeutic target. Iron is present in many enzymes, in addition to aconitase, and thus this is not the only potential mechanism but we focused on aconitase because of its importance in prostate cancer

Deferiprone (DFP) is an orally available iron chelator used in the clinic, primarily for the treatment of thalassemia and Friedreich ataxia. DFP readily enters cells and reaches the major intracellular sites of iron accumulation. In addition, this agent has been shown to impair the activity of m-Acon by removing iron from the mitochondria, and also to inhibit growth of some but not all cell lines.

2. **Keywords:** Deferiprone, aconitase, metabolism, tricarboxylic acid cycle, magnetic resonance

3. **Overall Project Summary:**

Overall Project Summary: DFP inhibited prostate cancer cell growth at low clinically achievable concentrations. DFP had a growth inhibitor effect in both prostate TRAMP C2 and Myc-Cap cell lines (two day incubation) (**Fig. 1A**) (Aim 1: subtasks 1,5, 6). Under regular growth conditions (0 µM DFP), the doubling times of TRAMP-C2 and Myc-CaP cells were 17.7±0.6 h and 15.2±2.8 h, respectively (**Fig. 1B**). The half maximal inhibitory concentration (IC$_{50}$) of DFP was about 50 µM (45.7±1.2µM for TRAMP C2 and 48.2±1.2 µM for Myc-Cap, **Fig. 1C,D**). The minimum DFP concentration tested that generated maximum cell growth inhibition effect was 100 µM. After this growth period (>48 h), cells in control wells (0 µM DFP) would become confluent, which for Myc-Cap cells led to detachment from the micro-plate surface. Still, additional studies incubating TRAMP C2 cells with DFP for three and five days showed similar growth inhibition effects and IC$_{50}$ values (49.4±1.1 and 60.5±1.1 µM, respectively). Deferiprone was also shown to inhibit cell migration in TRAMP C2 cells (Aim 1: subtask 9)

Metabolic changes associated with cell growth inhibition during the first 24h of exposure to DFP. (Aim 1: subtasks 6, part of 9, part of 10). We used our cell perfusion system to determine the effects of DFP on prostate cancer metabolism during the first 31h hours of incubation. Thus,
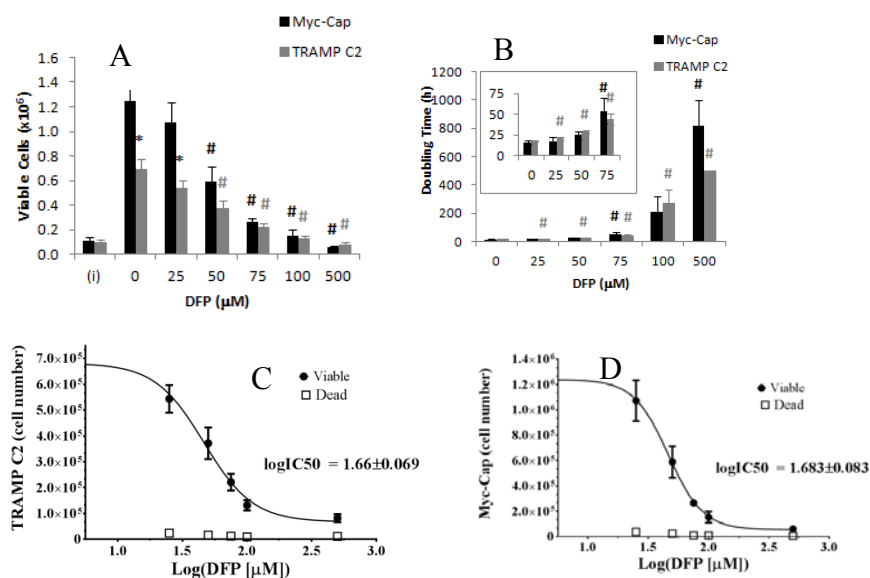
Figure 1 – TRAMP C2 and Myc-Cap cell growth inhibition after two day incubation with different concentrations of DFP (0, 25, 50, 75, 100, and 500 μM). Effect on cell growth (A) and doubling time (B), and log-growth curves (C) – IC50: Myc-Cap, 48.2±1.2 μM; TRAMP C2 45.7±1.2 μM. Error bars indicate the standard error of the means. (i) Number of viable cells at the time of medium change, for each cell line: Myc-Cap, $10.9 \pm 2.6 \times 10^4$.; TRAMP C2, $9.9 \pm 1.9 \times 10^4$.

TRAMP C2 cell were studied with and without 100 μM DFP in the perfusion medium. During that time, dynamic ^{31}P-MRS monitoring (**Fig. 2A**) showed that cells exposed to DFP internalize significantly less Pi from the medium after 17 h exposure (**Fig. 2B**). After 23 h exposure to the drug, a slight decrease was detected in total cellular bioenergetics (β-NTP, which is mostly ATP) and NAD$^+$(H) levels. At the same time, differences in choline metabolism became significant, as noted by the increase in the glycerophosphocholine to phosphocholine ratio (GPC/PC) were noted.
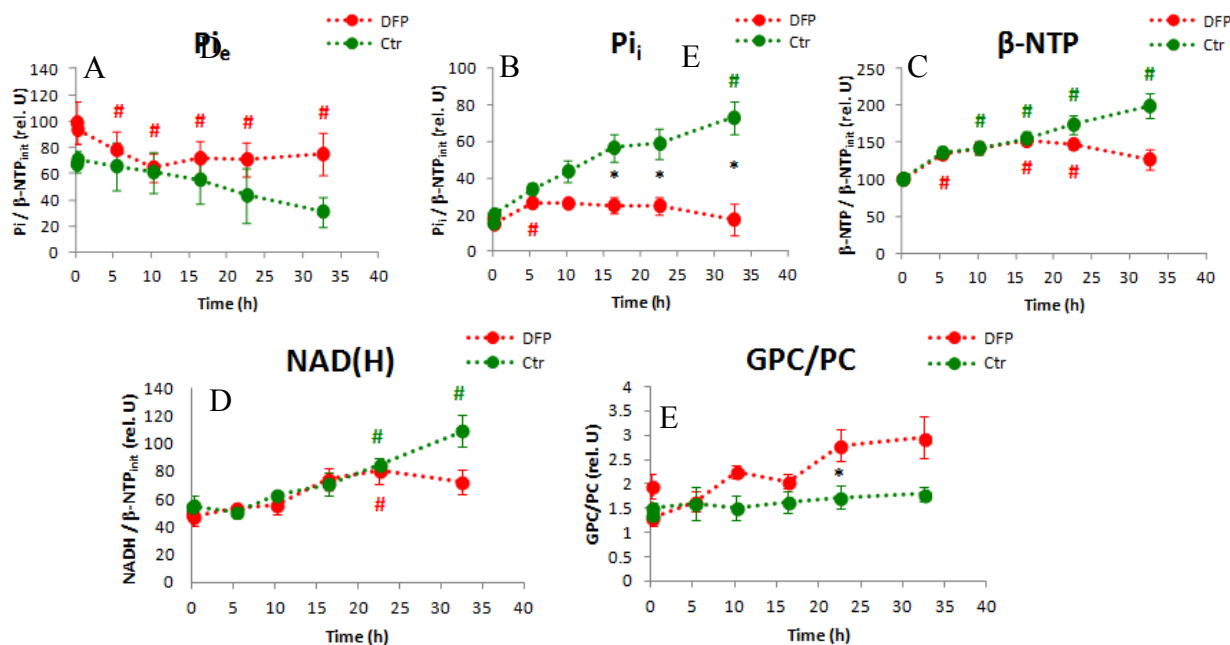

Figure 2 – Metabolic changes detected by ^{31}P-MRS during perfusion experiments of live TRAMP C2 cells. A, Representative ^{31}P-MRS profiles after 1 h (black) and at 31 h (green) of experiment time – major peak assignments displayed. B, Average time-course changes of inorganic phosphorous (extracellular, Pi$_e$; and intracellular, Pi$_i$), β-NTP (mostly ATP), total NAD (NAD$^+$ and NADH), phosphocholine to glycerophosphocholine ratio (GPC/PC), normalized to initial value of β-NTP (β-NTP$_{init}$); experiments carried out in regular medium (green) and in presence of 100 μM DFP (red). Error bars indicate the standard error of the means.
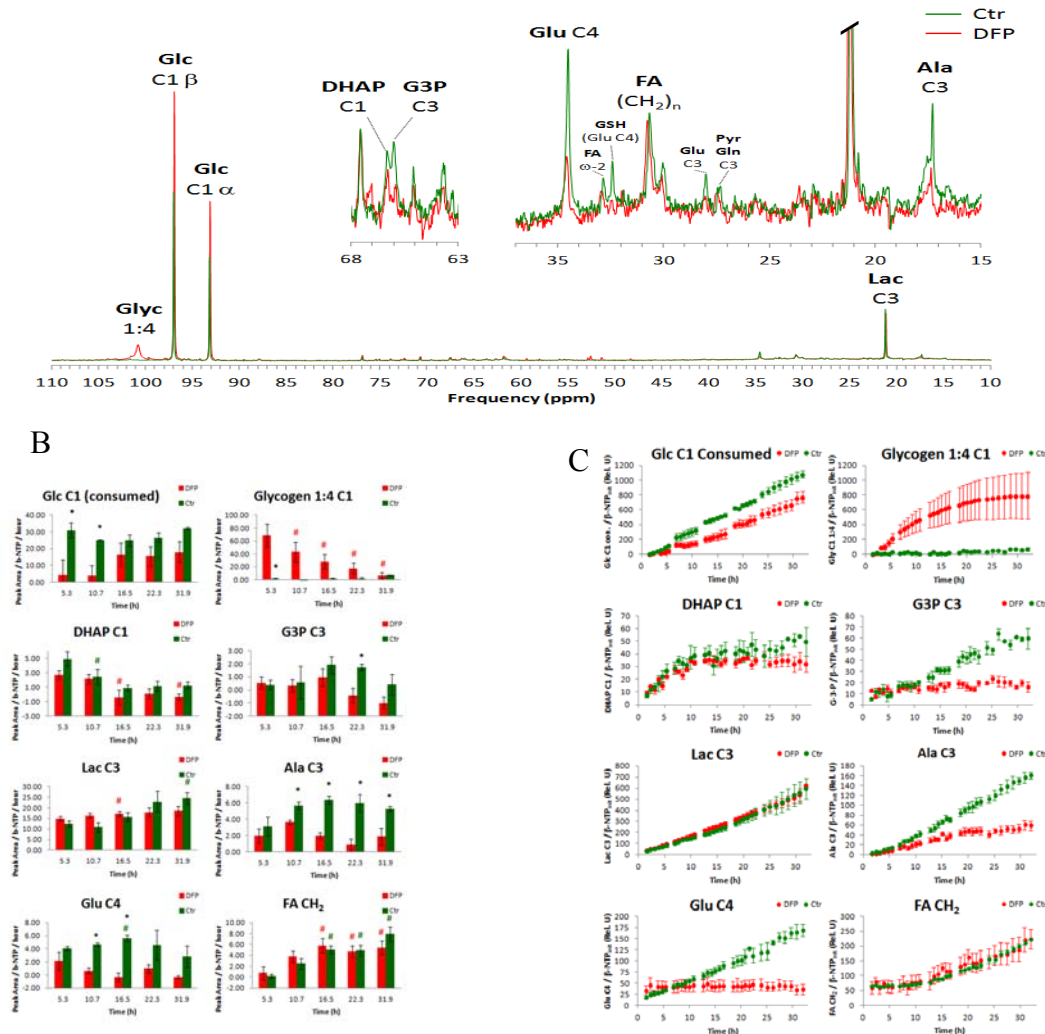
Figure 3 – Metabolic changes detected by ^{13}C-MRS during perfusion experiments with live TRAMP C2 cells. A, Representative ^{13}C-MRS profiles after 24 h of experiment time, both in regular medium (green) and in presence of 100 μM DFP (red) – major peak assignments displayed. B, Average time-course changes of 1-^{13}C-glucose consumption, 1:4-^{13}C-glycogen synthesis, 1-^{13}C-dihydroxyacetone phosphate (DHAP), 3-^{13}C-glycerol-3-phosphate (G3P), 3-^{13}C-lactate (Lac), 3-^{13}C-alanine (Ala), 4-^{13}C-glutamate (Glu), and (CH_2)-^{13}C-fatty acids (FA), normalized to initial value of β-NTP (β-NTP$_{init}$). C, Average metabolite synthesis (consumption) rates at different time intervals. Significant differences were detected between control and DFP-treated cells in 1-^{13}C-glucose consumption and synthesis of 1:4-^{13}C-glycogen, 3-[13]C G3P, 3-^{13}C-Ala and 4-^{13}C-Glu, but not in 1-[13]C- DHAP, 3-^{13}C-Lac and (CH_2)-^{13}C-FA synthesis.

DFP induced early changes in the glucose metabolism of prostate cancer cells. Parallel to ^{31}P-MRS, dynamic ^{13}C-MRS monitoring of TRAMP C2 cells perfused with 1-^{13}C-glucose (**Fig. 3A**) showed marked changes in the metabolism of glucose (**Fig. 3B**). Specifically, DFP led to a 6-fold decrease in 1-^{13}C-glucose uptake from the extracellular medium after 5h perfusion (**Fig. 3C**). At the same time, cellular incorporation of 1-^{13}C-glucose into glycogen was significantly higher in the presence of DFP. Along the glycolytic pathway in the cell cytoplasm, no significant changes were detected at any time in 1-^{13}C-glucose incorporation into dihydroxyacetone phosphate, although less was incorporated into glycerol-3-phosphate after 22 h exposure to DFP. Moreover, DFP did not induce any changes in glycolytic lactate synthesis rate but less 1-^{13}C-glucose was used for *de novo* alanine synthesis after 11 h exposure. As far as incorporation of ^{13}C labeling from glucose in the mitochondria, specific changes were noted in the TCA

(tricarboxylic acid) cycle, downstream of citrate. Thus, DFP essentially blocked the synthesis of 4-^{13}C-glutamate after 10 h exposure. On the other hand, no changes were detected at any time in the rate of incorporation of 2-^{13}C-acetate (derived from ^{13}C-labeled citrate exported to the cytosol) into fatty acids. These observations are indicative of m-Acon inhibition, independent of cytoplasmic aconitase, and therefore inhibition of the glucose-driven TCA cycle.
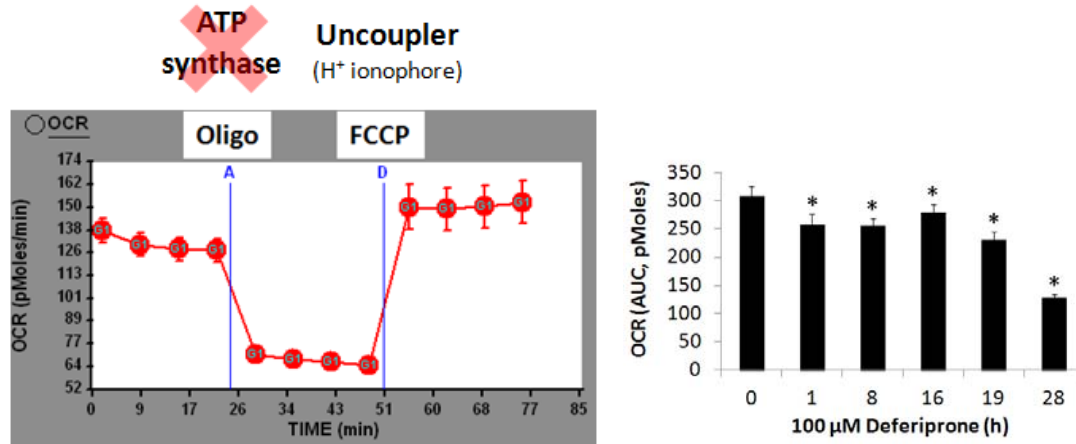

Fig. 4. (Left) Oxygen consumption rate **(**OCR) is coupled to ATP production in TRAMP C2 cells. Extracellular flux analysis experiments with the Seahorse system showed a marked decrease in OCR after inhibition of ATP synthase by oligomycin (Oligo). This did not affect the respiratory chain, since uncoupling the mitochondrial membrane (with FCCP) led to maximum OCR.   Right: **Effect of longer DFP incubation times in cellular OCR.** TRAMP C2 cells were seeded on 96-well microplates, as described in Methods section. Subsequently, the medium was replaced in selected wells with DFP (100 µM) containing medium, at different time points (at 6 h, 15 h, 18 h, 24 h, and 25 h). OCR levels were measured in each well 34 h after seeding the cells, using the Seahorse extracellular flux analyzer, as also described in Methods section. OCR significantly decreased with increasing DFP incubation times; specifically, 1.3-fold and 2.4-fold after 19 h and 28 h, respectively. This effect could be explained by a mixed contribution of lower cellular OCR and lower cell number (growth inhibition) with increasing DFP incubation time. The latter effect was not assessed in this experiment since the final quantification step (total protein per well) was not carried out.

DFP decreased the basal oxygen consumption rate in prostate cancer cells. To further investigate the effects of DFP on prostate cancer cells we carried out extracellular flux analysis experiments. Our preliminary studies showed that (i) oxygen consumption is coupled to ATP production in TRAMP C2 cells under normal growth conditions (**Fig. 4 Left**), and that (ii) OCR significantly decreases with increasing DFP incubation times (**Fig. 4 Right**). After 24h incubation with DFP in regular cell culture conditions, TRAMP C2 cells showed a significant decrease in OCR, with maximum effect observed at 100 µM DFP (1.7-fold decrease, Fig. 4, Right). This is consistent with the reduction in the glucose-driven TCA cycle, detected with the cell perfusion system (**Fig. 3B-C,** Glu C4). Moreover, no changes in basal ECAR levels were detected (**Fig. 5, Right**), which agrees with the lack of changes noted in the glycolytic lactate synthesis rate detected with the cell perfusion system (Fig. 3B-C, Lac C3).
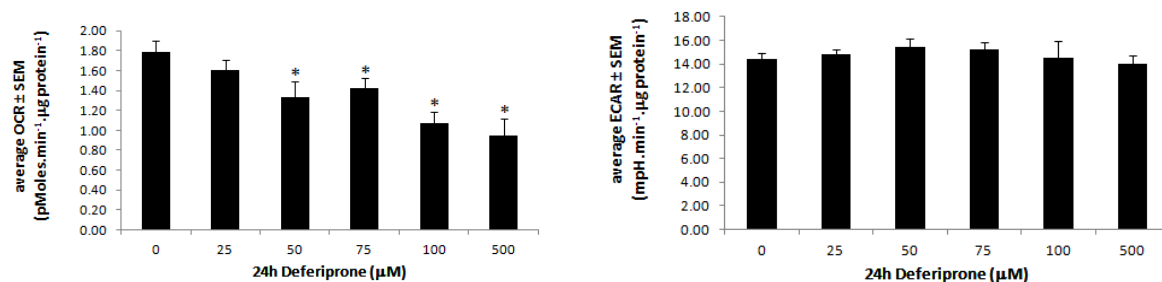
Figure 5 – Extracellular flux analysis in TRAMP C2 cells incubated with different concentrations of DFP. Left: OCR measurements.   Right: ECAR measurements. Error bars indicate the standard error of the means.

DFP reduced the expression of m-Acon in prostate cancer cells. The expression and the activity of m-Acon were investigated in both TRAMP C2 and Myc-Cap cells after 24h incubation with 100 µM DFP. Under regular growth conditions, the Western Blot expression of m-Acon was identical in both cell lines (**Fig. 6, Left**). DFP down-regulated the expression of m-Acon in TRAMP C2 cells (1.5-fold). Accordingly, the cellular activity of m-acon was 1.5-fold lower in the presence of DFP than in regular medium, in both TRAMP C2 and Myc-Cap cells (**Fig. 6, Right**). Altogether, these data are consistent with the TCA cycle inhibition (**Fig. 3B-C**) and the lower oxygen consumption rates (**Fig. 4, Left**) caused by DFP.
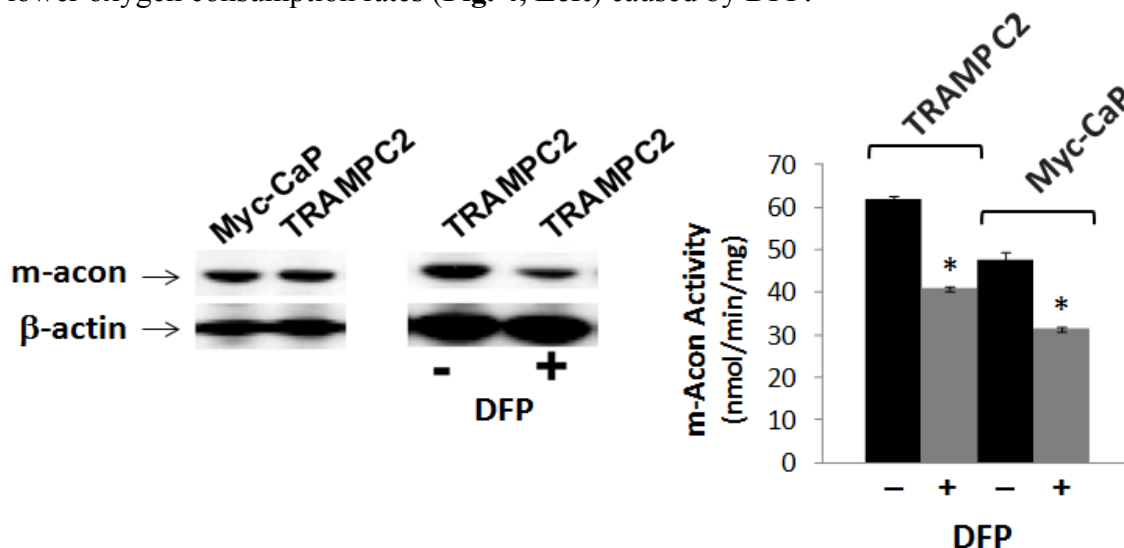

Figure 6 – Analysis of m-Acon in TRAMP C2 and Myc-Cap cells. Left: Western Blot expression. Right: Aconitase activity. Error bars indicate the standard error of the means.

We have submitted regulatory documents to the DOD for animal studies (Aim 1 subtask 2)

References:

1. Ibrahim AS, Edwards JE, Jr., Fu Y, Spellberg B. Deferiprone iron chelation as a novel therapy for experimental mucormycosis. The Journal of antimicrobial chemotherapy. 2006. Nov; 58(5):1070-3.

2. Simonart T, Noel JC, Andrei G, Parent D, Van Vooren JP, Hermans P, Lunardi-Yskandar Y, Lambert C, Dieye T, Farber CM, Liesnard C, Snoeck R, Heenen M, Boelaert JR. Iron as a potential co-factor in the pathogenesis of Kaposi's sarcoma? Int J Cancer. 1998. Dec 9; 78(6):720-6.

Key Research Accomplishments (bulleted list):
  A. Deferiprone was shown to inhibit prostate cancer cell growth in 2 independent cell lines
  B. Deferiprone was shown to inhibit prostate cancer cell migration in 2 independent cell lines
  C. Deferiprone was shown to inhibit tricarboxylic acid metabolism in perfused living cells; surprisingly it did not increase glucose consumption and lactate production
  D. The metabolic results were confirmed independently by measuring oxygen consumption rate and extracellular acidification rate
  E. The effect on metabolism was further explored by measuring aconitase activity and expression which were found to be significantly decreased.

Conclusions:
  A. Deferiprone is an active agent in treating prostate cancer in vitro
  B. A likely mechanism is that in chelating intracellular iron, it effects enzymatic activity

4. Impact: We have made significant progress in the in vitro studies. Excluding the difficulties discussed below with the cell transduction with shRNA to produce aconitase knockdown cells, we have made significant progress in Aim 1 (in vitro studies). Despite the difficulties with producing the knockdown cells, the more important studies are the Deferiprone studies for potential translation to patients, wherein we have proceeded at the required rate. This drug has been approved for patient use in other disease. In two cell lines (TRAMP C2 and MycCaP), we have found that the drug is active in inhibiting prostate cancer growth. Our studies of aconitase activity (with and without Deferiprone) are compatible with our hypothesis that it inhibits aconitase, although the knockdown studies will be more definitive for mechanistic reasoning.

5. Changes/Problems:

There has been a delay in producing aconitase knockdown cells. We are having problems with the technique. Therefore we have proceeded with the other studies (which had been planned to be performed in parallel with the studies on knockdown cells) so as not to fall behind the schedule of work. Thus task 7, 9, 10 are done partially

In the review of our IACUC protocol, it was severely criticized for putting Deferiprone in the drinking water which was felt to not be reliable. The IACUC has agreed that we should treat the mice (as done in many previous studies) with Deferiprone administered by oral gavage. DFP has been administered to mice at a range of 24mg/kg to 150 mg/kg twice a day. Ibrahim et al noted that 200mg/ kg/day was the maximum tolerated dose (MTD) (1) and Simonart et al suggested 150mg/kg/dose daily was safe for 5days/week x 1 month (2). The IACUC has agreed that we

should treat at 150 mg/kg/day x 1 month by oral gavage starting at a tumor volume of 25-50 mm3; longer durations may be considered depending on response.

We are currently injecting dead mice with prostate tumor cells for training for orthotopic tumor studies (Task 2, subtask 14). Under supervision of the IACUC, we are using dead mice and then plan to progress to in vivo studies. This was as proposed in the application. Our phantom studies suggest we may be forced to do the metabolic studies on subcutaneous tumors although this is not definitive and is being investigated in Year 2.

6. Products: Publications Abstracts and Presentations: None – manuscript in preparation (Aim 1, subtask 11). **TITLE:** Inhibition of prostate cancer growth by Deferiprone. **AUTHORS:** Rui V. Simões, Inna Serganova, Natalia Kruchevsky, Ellen Ackerstaff, George Sukenick, Ronald G. Blasberg, and Jason A. Koutcher

Inventions, Patents, and Licenses: Nothing to report

Reportable Outcomes: Nothing to report

Other Achievements: Nothing to report

7. Participants & Other Collaborating Organizations: None

8. Special Reporting Requirements: None

9. Appendices: None